

Applicants: Peter S. Linsley et al.
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Please amend the subject application as follows.

In the Title:

In accordance with 37 C.F.R. §§1.121(b)(1)(i)-(ii), please delete the title beginning on page 1 of the application and replace it with the following:

-- METHODS FOR GENERATING AND IDENTIFYING ANTIBODIES DIRECTED
AGAINST A B7 --

In the Specification:

Please amend the specification as follows:

Please replace the paragraph beginning at page 9, lines 21-25, with the following rewritten paragraph:

Figure 7a is a graph showing the effects of DR7-primed CD4⁺CD45RO⁺ T_h cells on differentiation of B cells into IgM secreting SKW B cells, as described in Example 2, *infra*.

Figure 7b is a graph showing the effects of DR7-primed CD4⁺CD45RO⁺ T_h cells on differentiation of B cells into IgG secreting CESS B cells, as described in Example 2, *infra*.

Please replace the paragraph beginning at page 9, lines 28-30, with the following rewritten paragraph:

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-- Figure 8a is a graph showing the effect of anti-CD28 and anti-B7 mAbs on the T_H-induced production of IgM by B cells as described in Example 2, *infra*.

92 Figure 8a is a graph showing the effect of anti-CD28 and anti-B7 mAbs on the T_H-induced production of IgG by B cells as described in Example 2, *infra*--

Please replace the paragraph beginning at page 9, lines 32-35, with the following rewritten paragraph:

93 -- Figure 9a is a diagrammatic representation of B7Ig protein fusion constructs as described in Example 3, *infra* (dark shaded regions = oncostatin M; unshaded regions = B7, stippled regions = human Ig Cyl).

Figure 9b is a diagrammatic representation of CD28Ig protein fusion constructs as described in Example 3, *infra* (dark shaded regions = oncostatin M; unshaded regions = CD28, stippled regions = human Ig Cyl).--

Please replace the paragraph beginning at page 11, line 8, with the following rewritten paragraph:

94 -- Recently, Freeman et al., (J. Immunol. 143 (8): 2714-2722 (1989)) isolated and sequenced a cDNA clone encoding a B cell activation antigen recognized by monoclonal antibody (mAb) B7 (Freedman et al., J. Immunol. 139:3260 (1987)). COS cells transfected with this cDNA were shown to stain by both mAb B7 and mAb BB-1 (Clark et al., Human Immunology 16:100-113 (1986), and Yokochi et al., (1981), *supra*; Freeman et al., (1989) *supra*; and Freedman et al., (1987), *supra*). The ligand for CD28 was identified by the experiments described herein, as the B7/BB-1 antigen isolated by Freeman et al., wherein the predicted amino acid sequence of amino acid 1-216 are:

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*DX
concl*

Gly	Leu	Ser	His	Phe	Cys	Ser	Gly	Val	Ile	His	Val	Thr	Lys	Glu	Val	1	5	10	15
Lys	Glu	Val	Ala	Thr	Leu	Ser	Cys	Gly	His	Asn	Val	Ser	Val	Glu	Glu	20	25	30	
Leu	Ala	Gln	Thr	Arg	Ile	Tyr	Trp	Gln	Lys	Glu	Lys	Lys	Met	Val	Leu	35	40	45	
Thr	Met	Met	Ser	Gly	Asp	Met	Asn	Ile	Trp	Pro	Glu	Tyr	Lys	Asn	Arg	50	55	60	
Thr	Ile	Phe	Asp	Ile	Thr	Asn	Asn	Leu	Ser	Ile	Val	Ile	Leu	Ala	Leu	65	70	75	80
Arg	Pro	Ser	Asp	Glu	Gly	Thr	Tyr	Glu	Cys	Val	Val	Leu	Lys	Tyr	Glu	85	90	95	
Lys	Asp	Ala	Phe	Lys	Arg	Glu	His	Leu	Ala	Glu	Val	Thr	Leu	Ser	Val	100	105	110	
Lys	Ala	Asp	Phe	Pro	Thr	Pro	Ser	Ile	Ser	Asp	Phe	Glu	Ile	Pro	Thr	115	120	125	
Ser	Asn	Ile	Arg	Arg	Ile	Ile	Cys	Ser	Thr	Ser	Gly	Gly	Phe	Pro	Glu	130	135	140	
Pro	His	Leu	Ser	Trp	Leu	Glu	Asn	Gly	Glu	Glu	Leu	Asn	Ala	Ile	Asn	145	150	155	160
Thr	Thr	Val	Ser	Gln	Asp	Pro	Glu	Thr	Glu	Leu	Tyr	Ala	Val	Ser	Ser	165	170	175	
Lys	Leu	Asp	Phe	Asn	Met	Thr	Thr	Asn	His	Ser	Phe	Met	Cys	Leu	Ile	180	185	190	
Lys	Tyr	Gly	His	Leu	Arg	Val	Asn	Gln	Thr	Phe	Asn	Trp	Asn	Thr	Thr	195	200	205	
Lys	Gln	Glu	His	Phe	Pro	Asp	Asn	210	215										

(Freedman et al., and Freeman et al., supra, both of which are incorporated by reference herein).-#

On page 13, please replace the first full paragraph with:

DS

-- In a preferred embodiment, DNA encoding the amino acid sequence corresponding to the extracellular domain of the B7 antigen, containing amino acids from about position 1 to about position 215, is joined to DNA encoding the amino acid sequences

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corresponding to the hinge, CH2 and CH3 regions of human Ig C γ 1, using PCR, to form a construct that is expressed as B7Ig fusion protein. DNA encoding the amino acid sequence corresponding to the B7Ig fusion protein has been deposited with the American Type Culture Collection (ATCC) at 10801 University Blvd., Manassas, Virginia 20110-2209 U.S.A., under the Budapest Treaty on May 31, 1991 and accorded accession number 68627.

On page 30, line 14, please replace the paragraph beginning "Cell Culture" with the following paragraph:

-- Cell Culture. T51, 1A2, 5E1, Daudi, Raji, Jijoye, CEM, Jurkat, HSB2, THP-1 and HL60 cells (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) were cultured in complete RPMITM medium (RPMITM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Dhfr-deficient Chinese hamster ovary (CHO) cells (Urlaub and Chasin, Proc. Natl. Acad. Sci., 77:4216-4220 (1980)) were cultured in Maintenance Medium (Ham's F12 MediumTM (GIBCO, Grand Island, NY) supplemented with 10% FBS, 0.15 mM L-proline, 100 U/ml penicillin and 100 μ g/ml streptomycin). Dhfr-positive transfectants were selected and cultured in Selective Medium (DMEMTM, supplemented with 10% FBS, 0.15 mM L-proline, 100 U/ml penicillin and 100 μ g/ml streptomycin).-v

On page 30, line 28, please replace the paragraph bridging pages 30 and 31, and beginning "Spleen B cells", with the following paragraph:

-- Spleen B cells were purified from Balb/c mice by treatment of total spleen cells with an anti-Thy 1.2 mAb (30H12) (Ledbetter and Herzenberg, Immunol. Rev. 47:361-389 (1979)) and baby rabbit complement. The resulting preparations contained approximately 85% B cells, as judged by FACS^R analysis following staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (TAGO). These

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cells were activated by treatment for 72 hrs with E. coli lipopolysaccharide (LPS, List Biological Laboratories, Campbell, CA) at 10 µg/ml in complete RPMI™.--

On page 31, line 5, please replace the paragraph "Monoclonal Antibodies" with the following:

D8
* Monoclonal Antibodies. Monoclonal antibody (mAb) 9.3 (anti-CD28) (ATCC No. HB 10271, Hansen et al., Immunogenetics 10:247-260 (1980)) was purified from ascites before use. mAb 9.3 F(ab')₂ fragments were prepared as described by Parham, in J. Immunol. 131:2895-2902 (1983). Briefly, purified mAb 9.3 was digested with pepsin at pH 4.1 for 75 min. followed by passage over protein A SEPHAROSE™ (beaded agarose) to remove undigested mAb. A number of mAbs to B cell-associated antigens were screened for their abilities to inhibit CD28-mediated adhesion. mAbs 60.3 (CD18); 1F5 (CD20); G29-5 (CD21); G28-7, HD39, and HD6 (CD22); HD50 (CD23); KB61 (CD32); G28-1 (CD37); G28-10 (CD39); G28-5 (CD40); HERMES1 (CD44); 9.4 (CD45); LB-2 (CD54) and 72F3 (CD71) have been previously described and characterized in International Conferences on Human Leukocyte Differentiation Antigens I-III (Bernard et al., Eds., Leukocyte Typing, Springer-Verlag, New York (1984); Reinherz et al., Eds., Leukocyte Typing II Vol. 2 New York (1986); and McMichael et al., Eds., Leukocyte Typing III Oxford Univ. Press, New York, (1987)). These mAbs were purified before use by protein A SEPHAROSE™ (beaded agarose) chromatography or by salt precipitation and in exchange chromatography. 8TA401 (Kuritani and Cooper, J. Exp. Med. 155:839-848 (1982)) (Anti-IgD); 2C3 (Clark et al., (1986), supra) (anti-IgM); Namb1, H1DE, P10.1, W6/32 (Clark et al., (1986) supra; and Gilliland et al., Human Immunology 25:269-289 (1989), anti-human class I); and HB10A (Clark et al., (1986), supra, anti-MHC class II) were also purified before use. mAbs B43 (CD19); BL-40 (CD72); AD2, 1E9.28.1, and 7G2.2.11 (CD73); EBU-141, LN1 (CDw75); CRIS-1 (CD-76); 424/4A11, 424/3D9 (CD77) Leu 21, Ba, 1588, LO-panB-1, FN1, and FN4 (CDw78); and M9, G28-10, HuLym10, 2-7, F2B2.6, 121, L26, HD77, NU-B1, BLAST-1, BB-1, anti-BL7, anti-

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HC2, and L23 were used as coded samples provided to participants in the Fourth International Conference on Human Leukocyte Differentiation Antigens (Knapp, Ed., Leukocyte Typing IV, Oxford Univ. Press, New York (1990). These were used in ascites form. mAbs BB-1 and LB-1 (Yokochi et al., (1981), supra) were also purified from ascites before use. Anti-integrin receptor mAbs P3E3, P4C2, P4G9 (Wayner et al., J. Cell. Biol. 109:1321-1330 (1989)) were used as hybridoma culture supernatants.--

On page 32, line 10, please replace the paragraph beginning "Immunostaining Techniques." with the following:

Immunostaining Techniques. For indirect immunofluorescence, cells were incubated with mAbs at 10 µg/ml in complete RPMI™ for 1 hr at 4°C. mAb binding was detected with a FITC-conjugated goat anti-mouse immunoglobulin second step reagent. For direct binding experiments, mAbs 9.3 and BB-1 were directly conjugated with FITC as described by Goding in Monoclonal Antibodies: Principles and Practices Academic Press, Orlando, FL (1983), and were added at saturating concentrations in complete RPMI™ for 1 hr at 4°C. Non-specific binding of FITC-conjugated mAbs was measured by adding the FITC conjugate following antigen pre-blocking (20-30 min at 4°C) with unlabeled mAb 9.3 or BB-1. Immunohistological detection of adherent lymphoblastoid cells was achieved using the horseradish peroxidase (HRP) method described by Hellstrom et al., J. Immunol. 127:157-160 (1981).-†

On page 34, line 15, please replace the two paragraphs beginning "CD28-Mediated Adhesion Assay" and "Labeled cells" with the following two paragraphs:

-- CD28-Mediated Adhesion Assay. Cells to be tested for adhesion were labeled with ⁵¹Cr (0.2-1 mCi) to specific activities of 0.2-2 cpm/cell. A mouse mAb having irrelevant specificity, mAb W1, directed against human breast carcinoma-associated mucin, (Linsley et al., Cancer Res. 46:5444-5450 (1986)), was added to the labeling reaction to a

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final concentration of 100 $\mu\text{g/ml}$ to saturate Fc receptors. Labeled and washed cells were preincubated in complete RPMITM containing 10 $\mu\text{g/ml}$ of mAb W1, and unless otherwise indicated, 10 mM EDTA. mAb 9.3 or mAb 9.3 F(ab')₂ was added to some samples at 10 $\mu\text{g/ml}$, for approximately 1 hr at 23°C.

Labeled cells ($1-10 \times 10^6/\text{well}$ in a volume of 0.2 ml complete RPMITM, containing EDTA and mAbs, where indicated) were then added to the CHO monolayers. Adhesion was initiated by centrifugation in a plate carrier (1,000 rpm, in a Sorvall HB1000 rotor, approximately 210 X g) for 3 min at 4°C. Plates were then incubated at 37°C for 1 hr. Reactions were terminated by aspirating unbound cells and washing five times with cold, complete RPMITM. Monolayers were solubilized by addition of 0.5 N NaOH, and radioactivity was measured in a gamma counter. For most experiments, numbers of bound cells were calculated by dividing total bound radioactivity (cpm) by the specific activity (cpm/cell) of labeled cells. When COS cells were used, their viability at the end of the experiment was generally less than 50%, so specific activity calculations were less accurate. Therefore, for COS cells results are expressed as cpm bound.--

On page 35, line 22, please replace the paragraph beginning "The effects of divalent cation depletion" with the following:

† The effects of divalent cation depletion on T51 cell adhesion to CD28⁺ and CD28⁻ CHO cells were examined. Preliminary experiments showed that EDTA treatment caused loss of CHO cells during washing, so the CHO cell monolayers were fixed with paraformaldehyde prior to EDTA treatment. Fixation did not significantly affect CD28-mediated adhesion by T51 cells either in the presence or absence of mAb 9.3. Monolayers of CD28⁺ and CD28⁻ CHO cells (1 to $1.2 \times 10^5/\text{cm}^2$ in 48 well plastic dishes) were fixed in 0.5% paraformaldehyde for 20 min at 23°C, washed and blocked in Complete RPMITM for 1 hr, then pre-incubated with or without mAb 9.3 or mAb 9.3 F(ab')₂ at 10 $\mu\text{g/ml}$ in Complete RPMITM for 1 hr at 37°C. T51 cells were labeled with

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Di 1 and
⁵¹Cr, preincubated with or without 10mM EDTA, added to CHO cells and cellular adhesion was measured. The results are presented in Figure 1. Mean and standard deviation (error bars) are shown for three replicate determinations.--

Di 2
On page 45, line 9, please replace the paragraph beginning "Culture medium" with the following:

-- Culture medium. Complete culture medium (CM) consisted of RPMI™ 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 2mM L-glutamine, 5×10^{-5} M 2-ME, and 10% FBS (Irvine Scientific).--

On page 55, line 31 through page 56, line 30, please replace the two paragraphs beginning "Cell Culture and Transfections" and "CHO cells expressing CD28," with the following paragraphs:

Di 3
Cell Culture and Transfections. COS (monkey kidney cells) were transfected with expression plasmids using a modification of the protocol of Seed and Anuffo (Proc. Natl. Acad. Sci. 84:3365 (1987)), incorporated by reference herein. Cells were seeded at 10^6 per 10 cm diameter culture dish 18-24 h before transfection. Plasmid DNA was added (approximately 15 µg/dish) in a volume of 5 ml of serum-free DMEM™ containing 0.1 mM cloroquine and 600 µg/ml DEAE Dextran™, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (approximately 2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM™ containing 10% FCS. At 24 h after transfection, culture medium was removed and replaced with serum-free DMEM™ (6 ml/dish). Incubation was continued for 3 days at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded.

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CHO cells expressing CD28, CD5 or B7 were isolated as described by Linsley et al., (1991) supra, as follows: Briefly, stable transfectants expressing CD28, CD5, or B7, were isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr⁻ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr, as described above in Example 1. Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1 μ M and were maintained in DMEMTM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate. CHO lines expressing high levels of CD28 (CD28⁺ CHO) or B7 (B7⁺ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting (FACS^R) following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr⁺ CHO) were also isolated by FACS^R from CD28-transfected populations. --

On page 56, line 33, please replace the paragraph beginning "Immunostaining and FACS^R Analysis," and continuing on page 57, with the following:

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-- Immunostaining and FACS^R Analysis. Transfected CHO cells or activated T cells were analyzed by indirect immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with murine mAbs 9.3 (Hansen et al., Immunogenetics 10:247 (1980)) or BB-1 (Yokochi et al., supra) at 10 μ g/ml, or with Ig fusion proteins (CD28Ig, B7Ig, CD5Ig or chimeric mAb L6 containing Ig Cy1, all at 10 μ g/ml in DMEMTM containing 10% FCS) for 1-2 h at 4°C. Cells were then washed and incubated for an additional 0.5-2h at 4°C with FITC-conjugated second step reagent (goat anti-mouse Ig serum for murine mAbs, or goat anti-human Ig Cy serum for fusion proteins (Tago, Inc., Burlingame, CA). Fluorescence was analyzed on 10,000 stained cells using a FACS IV^R cell sorter (Becton Dickinson and Co., Mountain View, CA) equipped with a four decade logarithmic amplifier.--

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On page 58, line 1, please replace the paragraph beginning "SDS Page" with the following:

9¹⁵
-- SDS Page. SDS-PAGE was performed on linear acrylamide gradients gels with stacking gels of acrylamide. Aliquots (1 µg) B7Ig (lanes 1 and 3 of Figure 10) or CD28Ig (lanes 2 and 4) were subjected to SDS-PAGE (4-12% acrylamide gradient) under nonreducing (-βME, lanes 1 and 2) or reducing (+βME, lanes 3 and 4) conditions. Lane 5 of Figure 10 shows molecular weight (M_r) markers. Gels were stained with Coomassie Brilliant Blue, destained, and photographed or dried and exposed to X-ray film (KodakTM XAR-5; Eastman Kodak Co., Rochester, NY) for autoradiography to visualize proteins.--

Please replace the three sequential paragraphs starting on page 59, line 27, with "Radiolabeling of B7Ig," and continuing to page 61, line 8, with the following three paragraphs:

7¹⁸
-- Radiolabeling of B7Ig. Purified B7Ig (25µg) in a volume of 0.25 ml of 0.12 M sodium phosphate, pH 6.8 was iodinated using 2 mCi ¹²⁵I and 10 µg of chloramin TTM. After 5 min at 23°C, the reaction was stopped by the addition of 20 µg sodium metabisulfite, followed by 3 mg of KI and 1 mg of BSA. Iodinated protein was separated from untreated ¹²⁵I by chromatography on a 5-ml column of SephadexTM G-10 equilibrated with PBS containing 10% FCS. Peak fractions were collected and pooled. The specific activity of ¹²⁵I-B7Ig labeled in this fashion was 1.5 x 10⁶ cpm/pmol.

B7Ig was also metabolically labeled with [³⁵S]methionine. COS cells were transfected with a plasmid encoding B7Ig as described above. At 24 h after transfection, [³⁵S]methionine (<800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to concentrations of 115 µCi/ml in DMEMTM containing 10% FCS and 10% normal levels of methionine. After incubation at 37°C for 3 d, medium was collected and used for purification of B7Ig as described above. Concentrations of [³⁵S]methionine-labeled B7Ig

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were estimated by comparison of staining intensity after SDS-PAGE with intensities of known amounts of unlabeled B7Ig. The specific activity of [^{35}S]methionine-labeled B7Ig was approximately 2×10^6 cpm/ μg .

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Binding Assays. For assays using immobilized CD28Ig, 96-well plastic dishes were coated for 16-24 h with a solution containing CD28Ig (0.5 μg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were then blocked with binding buffer (DMEMTM containing 50 mM BES, pH 6.8, 0.1% BSA, and 10% FCS) (Sigma Chemical Co., St. Louis, MO) before addition of a solution (0.09 ml) containing ^{125}I -B7Ig (approximately 3×10^6 cpm, 2×10^6 cpm/pmol) or [^{35}S]-B7Ig (1.5×10^5 cpm) in the presence or absence of competitor to a concentration of 24 nM in the presence of the concentrations of unlabeled chimeric L6 mAb, mAb 9.3, mAb BB-1 or B7Ig, as indicated in Figure 12. After incubation for 2-3 h at 23°C, wells were washed once with binding buffer, and four times with PBS. Plate-bound radioactivity was then solubilized by addition of 0.5 N NaOH, and quantified by liquid scintillation or gamma counting. In Figure 12, radioactivity is expressed as a percentage of radioactivity bound to wells treated without competitor (7,800 cpm). Each point represents the mean of duplicate determinations; replicates generally varied from the mean by $\leq 20\%$. Concentrations were calculated based on M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7Ig. When binding of ^{125}I -B7 to CD28⁺ CHO cells was measured, cells were seeded (2.5×10^4 /well) in 96-well plates 16-24 h before the start of the experiment. Binding was otherwise measured as described above.

On page 62, line 11, please replace the paragraph beginning "Cell separation and Stimulation" with the following:

D1
-- Cell Separation and Stimulation. PBL were isolated by centrifugation through Lymphocyte Separation MediumTM (Litton Bionetics, Kensington, MD) and cultured in 96-well, flat-bottomed plates (4×10^4 cells/well, in a volume of 0.2 ml) in RPMITM containing 10% FCS. Cellular proliferation of quadruplicate cultures was measured by

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uptake of [³H]thymidine during the last 5 h of a 3 day (d) culture. PHA-activated T cells were prepared by culturing PBL with 1 µg/ml PHA (Wellcome) for 5 d, and 1 d in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium™ before use.†

On page 69, line 1, please replace the paragraph beginning "RNA was prepared" with the following:

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-- RNA was prepared from stimulated PHA blasts by the procedure described by Chomczynski and Sacchi, Anal. Biochem. 162:156 (1987), incorporated by reference herein. Aliquots of RNA (20 µg) were fractionated on formaldehyde agarose gels and then transferred to nitrocellulose by capillary action. RNA was crosslinked to the membrane by UV light in a Stratalinker™ (Stratagene, San Diego, CA), and the blot was prehybridized and hybridized with a ³²P-labeled probe for human IL-2 (prepared from an approximately 600-bp cDNA fragment provided by Dr. S. Gillis; Immunex Corp., Seattle, WA). Equal loading of RNA samples was verified both by rRNA staining and by hybridization with a rat glyceraldehyde-6-phosphate dehydrogenase probe (GAPDH, an approximately 1.2-kb cDNA fragment provided by Dr. A. Purchio, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA).--

At page 78, line 21, please add SEQ. ID. NO. 8 as submitted by paper and on the enclosed diskette.